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Nitric oxide suppresses the energy transduction of plant mitochondria

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Introduction

Plant mitochondria are known to possess two respiratory electron transport pathways, i.e. the cytochrome and alternative pathways. The cytochrome pathway, which ends at cytochrome *c* oxidase (COX), is almost identical to the respiratory electron transport pathway in animal mitochondria. The alternative pathway, which ends at alternative oxidase (AOX), is unique in plants, fungi and parasitic protozoa. Although thermogenesis has been considered as a possible role for alternative pathway, the physiological significance of this non-energy producing pathway is not fully understood. Millar and Day (1996) have proposed that the alternative pathway may play an important role in preventing oxidative damage induced by nitric oxide (NO). Nitric Oxide (NO) is a free radical that can act as a signal messenger in animal cells (Packer 1996). It has been known that NO sometimes disturbs the energy transduction system in animal mitochondria by inhibiting the activity of COX (Brookes et al. 1999). In contrast to extensive knowledge on biochemistry and physiology of NO in animal systems, the source and role of NO in plants have been not yet confirmed. We have recently revealed that plant nitrate reductase (NR) is capable of converting nitrite to NO in the presence of NADH (Yamasaki, Sakihama 2000; Yamasaki 2000). NR is a key enzyme in nitrate assimilation pathway. By using the NR-catalyzed NO production system, here we demonstrate *in vitro* effects of NO on the energy transduction system of mitochondria. The results presented in this study provide substantial evidence to confirm that the plant alternative pathway is resistant to NO.

Materials and methods

Plant mitochondria were prepared from etiolated seedlings of mung bean (*Vigna radiata* L.). The activity of the electron transport system of mitochondria was determined from the rate of oxygen uptake measured with a Rank Brothers O_2

electrode. For monitoring membrane potential across the inner membrane of mitochondria, fluorescence measurements of safranine were carried out with a Shimadzu fluorometer (RF-5300, Shimadzu, Kyoto, Japan). The excitation and emission wavelengths were 520 and 570 nm, respectively. ATP synthesis activity was determined by luciferase ATP assay (ZS101, Yamato, Tokyo, Japan) with a luminometer (Gene light 55, Microtech Nichion, Shizuoka, Japan). The cytochrome pathway and alternative pathway were inhibited by antimycin A and *n*-propyl gallate (nPG), respectively. Antioxidant activity was determined from the rate of absorbance changes of DPPH (1,1-diphenyl-2-picrylhydrazyl) measured at 517 nm.

Results and Discussion

NO inhibits cytochrome pathway but does not affect alternative pathway
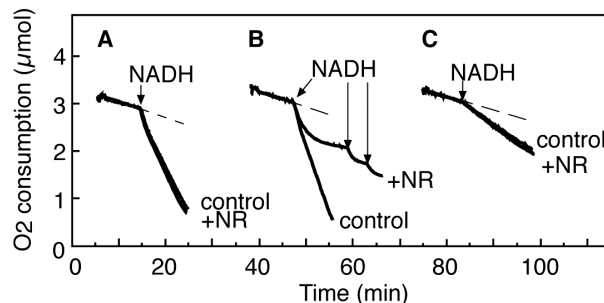
Fig.1. Oxygen uptake of mitochondria.

A: Both of the pathways are driven.

B: Cytochrome pathway is driven.

C: Alternative pathway is driven.

Figure 1 shows oxygen uptake activity of isolated mitochondria monitored with an O_2 electrode. To examine effects of NO on the respiratory electron transport activities, we used the NR/nitrite/NADH system, an enzymatic NO producing *in vitro* system (Yamasaki 2000). When only the cytochrome pathway was operational, oxygen uptake was strongly suppressed by the addition of NR (Fig. 1B). In contrast, oxygen uptake driven by the alternative pathway was not affected by the presence of NR (Fig. 1C). Using a nitrite/ascorbate nonenzymatic NO production system, Millar and Day (1996) found that the activity of oxygen uptake driven by the alternative pathway of *Glycine max* mitocondria was not inhibited by NO. Our results obtained with NR show a good agreement with this previous report.

Effects of NO on ATP synthetic activity in plant mitochondria

Proton motive force (*pmf*) generated across the inner membrane by the cytochrome pathway is the driving force for ATP synthesis in mitochondria. *Pmf* in mitochondria can be measured as the formation of membrane potential ($\Delta\psi$) across the inner membrane. Figure 2 shows changes in fluorescence intensity of safranine which can monitor the membrane potential formed across the inner membrane (Fig. 2A).
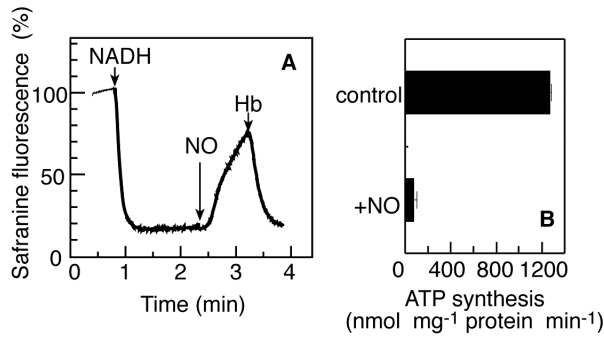
**Fig.2.** Inhibition of NO to respiration system.
A: The effect on membrane potential.
B: The effect on   ATP synthesis.

When the alternative pathway was inhibited by nPG, the addition of nitrite along with ascorbate rapidly diminished the fluorescence quenching of safranine.   Addition of hemoglobin, a strong scavenger of NO, recovered the absorbance of safranine to the steady-state level. Consistent with these $\Delta\psi$ responses, ATP synthesis in mitochondria was strongly suppressed while NO was produced from NR (Fig. 2B). These results have clearly shown that NO from NR not only inhibits the electron transport pathway but also affects the whole energy transduction system in plant mitochondria.

Antioxidant activity of an inhibitor of the alternative pathway

Salicylhydroxamic acid (SHAM) and nPG are well-known inhibitors of the alternative pathway. Although AOX has been identified as the site where these inhibitors act, the molecular mechanism of inhibition is still not clear. In antioxidant research, nPG is a
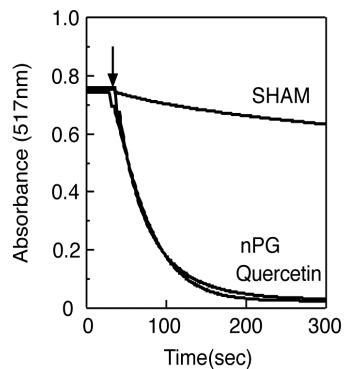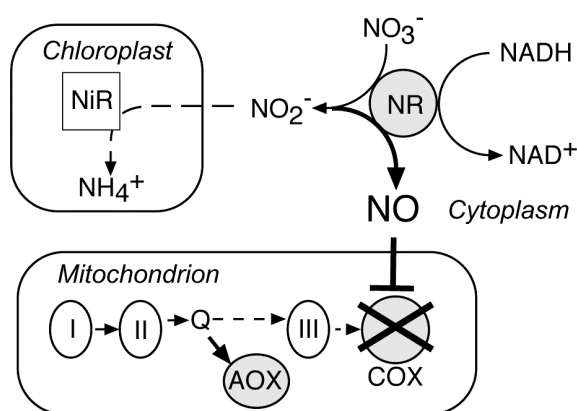

Fig.3. Antioxidant activity assay. SHAM, nPG and quercetin were added at the arrow indicated.

well-known antioxidant that can scavenge a range of radical species. Figure 3 shows the antioxidant activity of nPG and SHAM determined from DPPH radical scavenging activity. The addition of nPG or SHAM caused significant decrease in the absorbance. In particular, nPG rapidly abolished absorbance at a rate comparable to the antioxidant quercetin. Like nPG, quercetin inhibited the oxygen consumption driven by the alternative pathway, implying that not a specific structure but a radical scavenging activity would be necessary to inhibit the alternative pathway.

Physiological function of alternative pathway

NO production is catalyzed by NO synthase (NOS) in animal cells. Although it has been presumed that NO can be produced from NOS in plant cells as in animal cells, neither a protein nor a gene of NOS has been found yet. Normally, NR reduces nitrate to nitrite in the nitrogen assimilation pathway. However, NR potentially reduces nitrite to NO in situations that the concentration of nitrite is very high. In plant cells, the reduction of nitrite is catalized by chloroplastic nitrite reductase (NiR) that utilizes the photosynthetic power to reduce nitrite. Therefore, the accumulation of nitrite and subsequent NO production from NR would occur when NiR activity is inhibited under conditions where photosynthetic electron transport is not operational (Fig. 4). We consider it plausible that the alternative pathway is important in photoinhibiting conditons to prevent the production of active oxygen species when the cytochrome pathway is inhibited by NR-produced NO.

Fig.4. A hypothetical scheme for NO inhibition of respiratory chain.   Alternative pathway as a drain of electron.

Acknowledgement

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